



## Commentary

How to effectively treat acute leukemia patients bearing *MLL*-rearrangements ?Dieter Steinhilber<sup>a</sup>, Rolf Marschalek<sup>b,1,\*</sup><sup>a</sup> Institute of Pharm. Chemistry, Goethe-University, Frankfurt/Main, Germany<sup>b</sup> Institute of Pharm. Biology/DCAL, Goethe-University, Frankfurt/Main, Germany

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## ABSTRACT

Chromosomal translocations - leading to the expression of fusion genes - are well-studied genetic aberrations associated with the development of leukemias. Most of them represent altered transcription factors that affect transcription or epigenetics, while others - like BCR-ABL - are enhancing signaling. BCR-ABL has become the prototype for rational drug design, and drugs like Imatinib and subsequently improved drugs have a great impact on cancer treatments. By contrast, *MLL*-translocations in acute leukemia patients are hard to treat, display a high relapse rate and the overall survival rate is still very poor. Therefore, new treatment modalities are urgently needed. Based on the molecular insights of the most frequent *MLL* rearrangements, BET-, DOT1L-, SET- and MEN1/LEDGF-inhibitors have been developed and first clinical studies were initiated. Not all results of these studies have yet been available, however, a first paper reports a failure in the DOT1L-inhibitor study although it was the most promising drug based on literature data. One possible explanation is that all of the above mentioned drugs also target the cognate wildtype proteins. Here, we want to strengthen the fact that efforts should be made to develop drugs or strategies to selectively inhibit only the fusion proteins. Some examples will be given that follow exactly this guideline, and proof-of-concept experiments have already demonstrated their feasibility and effectiveness. Some of the mentioned approaches were using drugs that are already on the market, indicating that there are existing opportunities for the future which should be implemented in future therapy strategies.

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## 1. Introduction

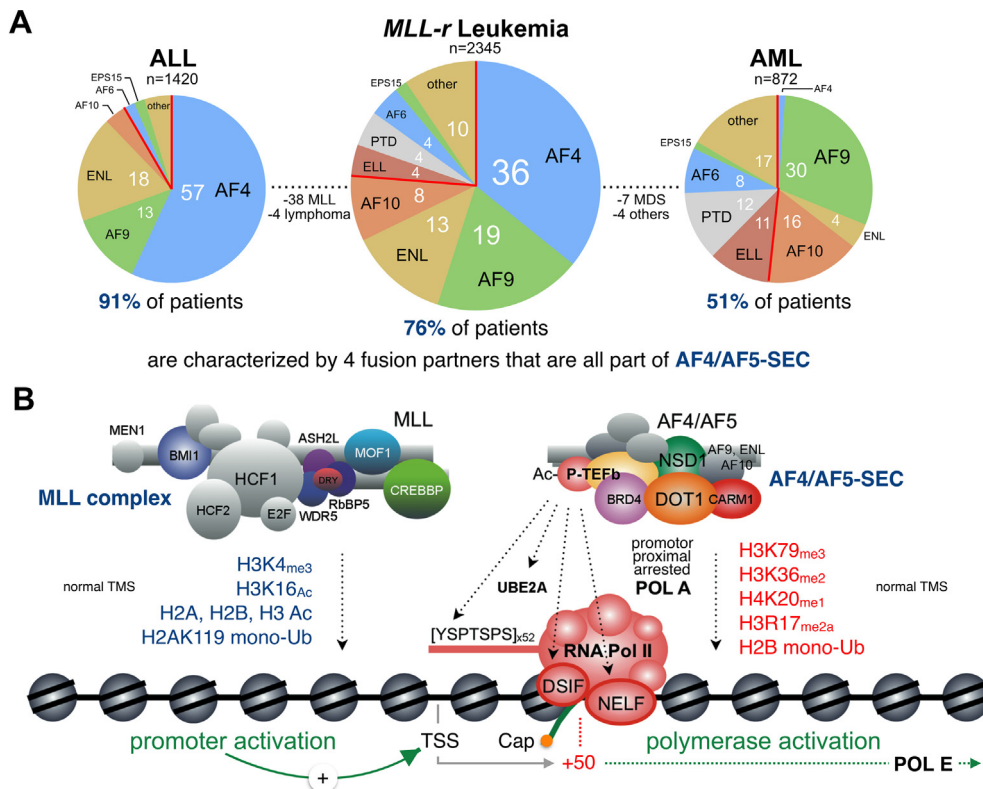
Acute leukemia bearing an *MLL*-rearrangement (*MLL-r*) is a distinct leukemia subgroup that needs much attention. While the overall survival of acute leukemias in pediatric patients reaches about 90%, *MLL-r* leukemia patients still display poor survival rates [1]. Despite their dismal clinical behavior, our knowledge about the pathological disease mechanism(s) exerted by some fusion proteins from distinct *MLL*-rearranged leukemias is quite good but does not yet translate into therapeutic success [2].

Basically, the *Mixed-Lineage-Leukemia* (*MLL*; or *HRX*, *ALL-1*, *KMT2A*) gene encodes a protein (500 kDa) which serves as a platform for the assembly of a multiprotein complex. The *MLL* complex influences gene transcription and chromatin by binding to target gene promoters, and reading and writing chromatin signatures ( $H3K4_{me3}$  and histone acetylation via bound proteins). Thus, the *MLL* complex generates a chromatin environment which enables active gene transcription (summarized in [2]). The *ALL-1 fused chromosome 4* (*AF4*; or *AFF1*) gene encodes another protein (178 kDa) which assembles again into a huge multiprotein complex. The *AF4* complex is necessary for the conversion of promoter arrested RNA polymerase II (POL A) into the elongating form (POL E). Thus, the *AF4* complex is a prerequisite for gene transcription in mammalian cells (summarized in [2]; see details also below).

In the past years different US and UK Universities – or their spin-offs – have developed a series of new inhibitors that can be potentially used to treat this disease entity [3–12]. All these drugs target either a druggable domain that is critical for the function of an *MLL* fusion or an associated protein. The targeted structures are either important for the onset or the maintenance of the associated

leukemia. It was also obvious from experimental results that *MLL* fusion proteins exert dramatic changes in the epigenetic program of the leukemia cells [13–18]. As a consequence of the presence of the *MLL* fusion protein and the changed epigenetic program, a highly stable gene expression signature is observed which can be visualized by heatmaps or even used for classifying *MLL-r* leukemia patients [19–24].

However, the molecular rearrangements observed in *MLL-r* leukemia patients are rather complex and require a more differentiated view. Recently published data from our own lab about the *MLL* recombinome display a picture with a total of 100 *MLL-X* fusions (84 in-frame – 16 out-of frame), and 247 *X-MLL* fusions (32 in-frame – 215 out-of-frame) [25]. About 45% of these fusions have been characterized in single leukemia patients. By contrast, most of the ALL (~90%) and about half of the AML patients are diagnosed with only 4 different *MLL* translocations (see Fig. 1A). They were encoding exactly those fusions which have been characterized intensively over the past decades: *MLL-AF4*, *MLL-AF9*, *MLL-ENL*, *MLL-AF10* and *AF4-MLL*. Interestingly, the proteins *AF4*, *AF9*, *ENL* and *AF10* are all integral part of the *AF4* super elongation complex (SEC) [26,27]. Besides the mentioned proteins, the kinase *P-TEFb*, the bromodomain protein *BRD4* and the histone methyltransferases *DOT1L*, *NSD1* and *CARM1* are all integral part of this complex (see Fig. 1B, right side) [28]. This *AF4* SEC has several important functions for gene transcription: (1) the *BRD4* protein exhibits bromo domains that read acetylated histone marks which in turn allows to recruit *P-TEFb* alone or the *P-TEFb* containing *AF4* SEC to active chromatin regions; (2) phosphorylation of the CTD domain of RNA polymerase II, *DSIF* and *NELF* converts *POL A* (promoter proximal arrested) into *POL E* (elongating



**Fig. 1.** (A) Distribution of translocation partner genes in *MLL-r* leukemia, separated by ALL and AML. When focussing only on the fusion partners *AF4*, *AF9*, *ENL* and *AF10*, 91% of all ALL and 51% of all AML patients have been diagnosed with these fusions. Diagnosed patient were n = 2345. (B) The “transcriptional memory system” of our cells. Due to the action of *MLL* and *AF4* multiprotein complexes, all transcribed genes are marked by distinct chromatin marks that allow a given cell to remember which genes are necessary to be transcribed in order to maintain cell identity. Therefore, any interference with these wildtype proteins may cause a “memory loss” with unpredictable side effects.



**Table 1**  
Published efforts to selectively inhibit oncogenic fusion genes. This table summarizes the strategies of different labs (named below the Table) and to selectively inhibit either of the two fusion proteins MLL-AF4 or AF4-MLL (lines 1 and 4). The effect of the therapeutic intervention on the cognate wildtype proteins in t(4;11) translocations (lines 2 and 3) are shown as well. Expected therapeutic effects are depicted in green, interference with wildtype proteins in red, while “no effect” is marked in black.

Publication Ref	2004-2014 30,31	2011 41	2004-2015 42	2014-2016 32,43,67	2005-2011 44-46	2004-2014 7	2010-2013 3,4,8,9	2014 10	2012-2015 5,6,11,12
	blocking binding of ENL and AF9	blocking MLL FYRN/FYRC	blocking Taspase1	class I HDACi	Antisense oligos	DOT1L-i	BET-i	SET-i	MEN1 LEDGF-i
MLL-AF4	inactivation	no effect	no effect	full inactivation	temporary inactivation	inactivation	partially effective due to AF4 SEC recruitment	no effect	inactivation
MLL	no effect	no effect	no effect	activation	near no effect	no effect	no effect	inactivation	inactivation
AF4 (SEC)	inactivation	no effect	no effect	partial inactivation	near no effect	inactivation	inactivation	no effect	no effect
AF4-MLL	no effect	protein degradation	protein degradation	partial inactivation	no effect due to long half-life of AF4-MLL	inactivation	no effect	inactivation	no effect
	Hemenway lab	Marschalek lab			Heidenreich lab	Epyzyme Celgene Pollock lab	Knapp Bradner Nicodème Kouzarides labs	Dou lab	Grembecka Cierpicki lab

inhibition concepts affect also the cognate wildtype proteins (see below). Since MLL – as well as some of the major fusion partner proteins – are involved in fundamental biological processes, we need to be more cautious. The holy grail will be the identification of novel ways to selectively inhibit the MLL fusion protein, while preserving the functions deriving from the corresponding wildtype proteins.

All efforts to selectively target MLL fusion proteins have been summarized in Table 1. The overexpression or transduction of a short peptide deriving from the AF9/ENL binding module of AF4 family members prevents binding of AF9 or ENL [30,31]. The recombinant expression of FYRN or FYRC-derived peptides (~50 amino acids) disturbed the heterodimerization of the AF4-MLL-N with MLL-C and caused the degradation of the AF4-MLL fusion protein [41]. Similarly, the inhibition of endogenous Taspase1 by transfected dnTASP1 also caused the proteasomal degradation of the AF4-MLL fusion protein [42]. Very recently, HDAC inhibition was shown to have a profound effect on the MLL-AF4 fusion protein, while concomitantly activating the endogenous MLL protein. Mechanistically, HDAC treatment caused a displacement of MLL-AF4 by endogenous MLL at several target gene promoters (*ALOX5* and *HOXA* genes) [32,38,43]. Proof-of-concept experiments using antisense oligos against the MLL-AF4 fusion gene transcript were already successfully performed 10 years before [44], while the same approach aiming to target the AF4-MLL fusion transcript has failed because of the extremely long half-life of the AF4-MLL fusion protein complex (~96 h) in contrast to the short-term effects of siRNA-mediated knockdowns (~48 h) [45,46].

## 2. Why should wildtype proteins be preserved and not targeted in MLL-r leukemia?

MLL and AF4 share the critical function to represent a molecular nexus for the formation of high molecular weight protein complexes that are crucial for the regulation of gene transcription and the chromatin status. The chromatin modifying abilities of both protein complexes in conjunction with PAF1C are essential

for the “transcriptional memory system” (TMS) of our cells (see Fig. 1B). This is due to the different epigenetic imprints at active promoters (MLL) and the transcribed gene bodies (AF4). Basically, the actions of both protein complexes can be described as “highlighting” the transcribed genes in the context of surrounding chromatin (repressed or active). As summarized in Fig. 1B, the MLL complex exerts different histone modifying activities that mark active promoters (H3K4<sub>me3</sub>, H3K16<sub>Ac</sub>, etc). The AF4 complex usually cooperates with PAF1C to release promoter-proximal arrested “POL A” [47]. The conversion of “POL A” into elongating “POL E” is accompanied by P-TEFb-mediated (1) destruction of NELF, (2) activation of DSIF and (3) CTD-phosphorylation at Ser-2 residues. Moreover, transcribed gene regions get modified with certain signatures (H3K79<sub>me3</sub>, H3K36<sub>me2</sub>, H4K20<sub>me1</sub>, etc). Due to these mechanisms, all genes in a given cell are marked-up in the chromatin to maintain their transcriptional activity.

Any drug which targets functional activities of either of these two protein complexes, MLL or AF4, could potentially lead to a “memory loss” or to a counterregulation by other available epigenetic modifiers. Such drugs applied to healthy cells may simply result in apoptosis, but in case of strongly selected cells – like cancer cells – it may even enhance their capacity to adapt to the new situation and to become resistant against the given treatment [48]. Cancer cells usually overwrite the existing “TMS” by using these fusion proteins and establish an “oncogenic TMS” (OTMS). Such a new OTMS can be visualized by robust gene expression profiles and includes many new functions and plasticity. It is also important to keep in mind that leukemogenic fusion proteins seem to act dominantly over their wildtype counterparts, but may require their functions (see Fig. 2).

## 3. Alternative solutions

As already mentioned above, the literature provides a series of interesting possibilities that can be used for future treatment approaches. Some of them are depicted in Fig. 2, putative approaches may include: (1) to interfere with the AF4 SEC



hijacking process; (2) to prevent the intramolecular dimerization process (FYRN/FYRC or Taspase1 inhibition) which causes a rapid proteasomal degradation of the AF4-MLL fusion protein; (3) to functionally inactivate MLL-AF4 and to replace it by an activated MLL by using class I HDACi.

#### 4. Interfering with the AF9/ENL-AF4 interaction

The Hemenway lab has published a series of articles where they have demonstrated the effectiveness of a competitive peptide (PFWT = Pen-LWVKIDL DLLSRV) either applied as synthesized peptide or recombinantly expressed to disrupt the interaction of AF4 with AF9 or ENL, respectively. The efficacy was proved by *in vitro* and *in vivo* experiments and impaired cell growth of t(4;11) and t(5;11) cells expressing the MLL-AF4 or MLL-AF5 fusions, respectively. In addition, apoptosis was induced in a significant portion when investigated after 24 h of treatment (>80%) [30]. Even in a mouse model the use of a synthetic peptide with modified amino acids that has been fused with a TAT transduction domain (SPK111) was shown to display therapeutic efficacy against *i.v.* injected tumor cells bearing the *MLL* translocations t(4;11), t(9;11) and t(11;19), while not affecting REH or MOLT-4 control cells [31]. These proof-of-concept papers have already demonstrated that the hijacking mechanism of MLL fusion proteins can be impaired, particularly in cases when MLL-AF4, MLL-AF9 and MLL-ENL are present.

Are there any restraints? Maybe, because the PFWT peptide (used as a therapeutic peptide) will also disrupt the interaction of AF9 or ENL with the wildtype AF4 complex. Whether this has any effect on the function of the AF4 complex has not yet been investigated, but based on a recent study [49] it can be assumed. Overexpression or downregulation of AF4 was strictly correlated with the overall abundance of mRNA in cells. Impairing AF4 functions strongly influences the capability of a cell to cope with adverse situations. We were able to knockdown AF4 only to a level of about 40%, otherwise cells were disappearing. Therefore, a treatment of cells by such a peptide (or derivatives thereof) might result in cellular apoptosis. Preventing the binding of AF9 or ENL may compromise a central function of AF4 complex, namely transcriptional elongation, since both proteins bind via their YEATS domain to DOT1L [50]. However, a recent study of our lab has shown that DOT1L can also interact with Cyclin T1 [51]. Therefore, it will be necessary to analyze the composition of the AF4 complex in the absence or presence of the PFWT peptide in order to understand the impact of such a treatment.

#### 5. Interfering with the FYRN/FYRC interaction of MLL

Similarly, we have shown some years ago that the AF4-MLL fusion protein exhibits an important self-destruction mechanism [52]. This mechanism could be used to get rid of this oncoprotein which by itself was sufficient to cause ALL in mice [53]. Two different strategies have been applied. The first approach was to target the assembly of the AF4-MLL fusion protein. The assembly of the AF4-MLL complex is critically dependent on the cleavage by Taspase1 [54], which occurs at CS1 (QVD-GADD; minor cleavage site) and/or CS2 (QLD-GVDD; major cleavage site). This allows the formation of an intermolecular interaction of the resulting protein fragments via the FYRN and FYRC domains. These FYRC/N domains have been mapped in different labs [55,56], but the minimal interaction interface is composed by amino acids 1991-2104 (FYRN) and 3651-3752 (FYRC) [41] which fitted perfectly to the published FYRN/FYRC domain structure of the TGF $\beta$  regulator 1 protein [57]. When FYRN- or FYRC-derived peptides (~50 amino acids) were additionally expressed in AF4-MLL expressing cells, they disabled

the interaction between AF4-MLL-N and MLL-C and led to a rapid destruction of both proteins, respectively [41]. Destruction of both proteins could be inhibited by adding the proteasome inhibitor MG132, indicating that the assembly of the highly stable complex [28] is a necessary step for the AF4-MLL fusion protein to exert its oncogenic function.

#### 6. Inhibition of Taspase1 functions

In a second approach, we studied Taspase1 at the molecular level by using a point-directed mutagenesis approach. Experimental analyses of the resulting mutant proteins revealed a series of important amino acid positions which are crucial for Taspase1 activity. Moreover, it allowed us to design a dominant-negative Taspase1 (dnTASP1) which – when expressed together with endogenous wildtype Taspase1 – is able to bind and inactivate the wildtype Taspase1 [42]. We used the expression of either wildtype Taspase1 or dnTASP1 to demonstrate that the inhibition of AF4-MLL cleavage by Taspase1 is leading to the self-destruction of the AF4-MLL oncoprotein. Otherwise the AF4-MLL assembles into a highly stable protein complex with an estimated half-life of about 96 h. To this end, either the inhibition of Taspase1 or interfering with the FYRN and FYRC interaction specifically leads to a rapid proteasomal degradation of the AF4-MLL fusion protein. This is not true for the wildtype MLL protein, because the abundance of MLL is controlled by different degradation pathways [58,59].

A potential caveat could derive from the fact that MLL, MLL4 and TFIIA are highly specific targets of Taspase1-mediated cleavage. However, it has already been shown that TFIIA cleavage is not required for TFIIA functions, rather fine-tunes transcriptional processes [60]. In addition, the Taspase1 *k.o.* mice were viable, indicating that both MLL and MLL4 are not essentially impaired when Taspase1 is absent [61]. It seems that the MLL protein even without Taspase1 processing is still capable to form an FYRN and FYRN interaction in order to assemble into a functional complex [62]. We therefore conclude that Taspase1 is a conditional oncoprotein in case of t(4;11) leukemia and that Taspase1 is a valid target structure for the development of novel drugs.

#### 7. Use of class I HDAC inhibitors

Finally, we propose that class I HDCA inhibitors should be introduced into clinical trials. This finding came from experiments using the *ALOX5* gene, a target of the MLL protein. *ALOX5* encodes 5-Lipoxygenase (5-LO), a key protein for host defence and inflammatory reactions in humans. 5-LO is mainly expressed in leukocytes and its expression is strongly upregulated during myeloid cell differentiation by calcitriol and TGF- $\beta$  [63]. 5-LO has been linked with leukemia as it was found that its knockout impairs leukemia stem cells [64,65]. Regulation of 5-LO mRNA expression occurs at the level of transcript initiation as well as elongation. Transcriptional initiation is mediated by multiple SP1 binding sites whereas signaling via the vitamin D receptor stimulates transcript elongation [66]. In case of *ALOX5*, transcriptional elongation is however controlled not at +50 rather than about 40–80 kb downstream of the TSS in an intronic region, where vitamin D receptor activation stimulates phosphorylation of POL-II at Ser2 in the distal part of the *ALOX5* gene and promotes elongation of the 5-LO transcript. We have used this interesting gene system to study the functions of MLL-AF4 (transcription initiation) and AF4-MLL (transcriptional elongation) [32,43]. MLL-AF4 is strongly enhancing the transcription of this promoter in reporter gene assays by up to 50-fold due to binding to the SP1 transcription factors, while AF4-MLL overwrites the elongation control mechanism of the

*ALOX5* gene. When TSA (pan-HDACi) was applied, it turned out that the H3K4<sub>me3</sub> signatures were increasing, while MLL-AF4-mediated transcription was dramatically dropping down towards the activity of endogenous MLL. Therefore, a series of different HDAC inhibitors were tested to find out which HDACs are presumably of importance. It turned out that only class I HDAC inhibitors (Entinostat, Droxinostat and Mocetinostat) were scoring. By using ChIP experiments we were able to demonstrate that inhibition of class I HDACs inactivates the MLL-AF4 fusion protein, while the endogenous MLL protein becomes activated. This led to a displacement of MLL-AF4 by endogenous MLL at the *ALOX5* target gene promoter. Similarly, this was shown later also for the *HoxA9* and *HoxA10* promoter when using TSA [38]. Inhibition of class I HDACs was sufficient for this “MLL replacement effect”. As a result, transcription becomes normalized back to physiological levels. Co-IP experiments revealed that MLL-AF4 is indeed associated with HDAC1 and HDAC2, however, the precise mode of action and why HDACi “inactivates” rather than “activates” MLL-AF4 is still elusive.

That HDAC inhibition is presumably beneficial was first published some years ago by the group of Ronald Stam, who combined gene expression profiles with a connectivity map (cmap) analysis [67]. This paper suggested HDAC inhibition to be beneficial for treatment of t(4;11)-translocated cells. They already tested TSA, SAHA, LHB589, VPA, FK228 and MS-275 (Mocetinostat). A differential killing of t(4;11) cells versus non-t(4;11) leukemic cells was achieved only with TSA, while all other tested drugs seemed to be not very specific. However, when t(4;11)-translocated cells were compared with normal bone marrow cells then TSA, LHB589 and MS-275 were quite successful, indicating that no severe side effects on normal hematopoiesis should be expected when up to 500 nM of either of these substances was applied. While this paper revealed HDACi as potential drugs, no mechanistic insight was provided.

Another interesting member of class I HDACs is HDAC3. HDAC3 is quite necessary for a full activation of P-TEFb (heterodimer of Cyclin T1 and CDK9). P-TEFb becomes activated by a T-loop phosphorylation at T186 which allows *per se* binding of ATP in the binding pocket. However, P300 and GCN5 are acetylating the residues K44 and K48/K49 of CDK9, thereby inhibiting ATP binding. HDAC3 is necessary to remove the acyl-group from K44, which allows binding of ATP next to the catalytic site [68]. Thus, HDAC3 inhibition might reduce P-TEFb kinase activity, which in case of t(4;11) translocations may inhibit the activity of the AF4-MLL fusion protein. Thus, the same drug is not only impairing functions of MLL-AF4, but also of AF4-MLL. In addition, it functionally activates the endogenous wildtype MLL. Therefore, class I HDACi seems to be a perfect treatment strategy to kill t(4;11) leukemia cells.

Another recent report demonstrated that HDAC3i is impairing the AKT signaling pathway and increases the chemosensitivity of leukemic cells [69]. This could be another argument for the introduction of HDACi into clinical trials.

Potential caveats of HDAC inhibitor treatments are the many off-target effects on cellular proteins that are, however, dose-dependent and difficult to analyze. Side effects of HDACi treatments have been described (e.g. diarrhea, thrombocytopenia), but are clinically manageable.

## 8. Selective degradation

As already outlined above, the degradation of oncoproteins is a highly specific intervention strategy. This is not only true for MLL fusions but can also be observed for other leukemogenic fusions: interfering with the coiled-coil domain of BCR-ABL or the BTB/POZ domain of PML-RARA causes a rapid proteasomal degradation of both fusion proteins [70–72]. Similarly, the disruption

of tetramerization domain of RUNX1-ETV6 (NHR2-domain) by a small drug has the same effect and abolishes all oncogenic activities [73]. Therefore any strategy leading to a rapid turnover of a distinct oncoprotein seems to be a valid venue and should be consequently pursued.

A recent paper demonstrated that the stabilization of the endogenous MLL protein is helping to prevent actions of the corresponding MLL fusion proteins [74]. In that case an IRAK inhibitor was used to block the degradation of endogenous MLL. The results of this study are in line with the above mentioned concept, that any selective strategy that kills the oncoprotein, and/or, leads to the enhancement of the endogenous protein could be of benefit for patient treatment.

Therefore, the recently described PROTAC technology is quite attractive for future developments. This technology is based on the knowledge about known protein-protein interactions. If a binding partner is known, amino acid sequences thereof can be fused to a degradation box that attracts the corresponding E3 ligases for ubiquitination. However, this happens only after administration of a specific drug which in turn causes the proteasomal degradation of the target protein (for latest review see ref [75]). In case of the t(4;11) fusion protein there have been a lot of protein-protein interactions described (MEN1, LEDGF1, GADD33, CYP33, BMI-1, DOT1L, NSD1, CDK9, CYCT1, AF4, AF9, ENL, etc.) which could be potentially used as target structure for fusing with an E3-ligase attractor signal. Even the combination of two different targeting peptides spaced by an E3-ligase attractor signal to selectively target oncogenic fusion proteins might be quite useful to fulfill the goal of selectivity. All these suggestions may help to find new forms of treatment for MLL-r leukemia patients.

### 8.1. Outlook

This manuscript tries to tie up some interesting findings of many labs that are of great value for new options in the treatment of leukemia patients that suffer from MLL-rearrangements. This paper deals with data obtained with 4 major MLL fusions (MLL-AF4, MLL-AF9, MLL-ENL, MLL-AF10). Many investigators have found interesting strategies to interfere with the oncogenic functions deriving from the fusion proteins. Obvious strategies, like e.g. inhibition of DOT1L, have however failed due to therapy resistance. There are still studies missing using inhibitors against MEN1 or the SET domain, but it is foreseeable that these studies will run into similar problems.

A solution to these emerging problems could be to specifically target only the fusion proteins. This paper has summarized such efforts and has shown that it is principally possible, but it will require tremendous efforts to bring them into clinical trials. Current efforts are summarized in Table 1, indicating their effects on wildtype and fusion proteins. Based on our knowledge and the hints provided by Table 1, several of these strategies could work, but need to be tested *in vitro* and in clinical trials.

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